THE MORPHOLOGY AND DEVELOPMENT OF CERTAIN PYRENOMYCETOUS FUNGI.

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(WITH PLATES XIV-XVI)

THE study of the morphology and development of the ascomycetous fungi comprises four essential questions: (1) sexuality; (2) structure of sporocarp; (3) origin and development of spore; (4) presence, structure, and behavior of nuclei. The literature includes the results of many valuable investigations dealing with one or more of these questions throughout the great class Fungi. The thoroughness and accuracy of this work has advanced steadily with the improvement of methods, and much of the earlier work has been disproved later. In the meantime two opposed schools have arisen, one maintaining the sexuality of the higher fungi and their relation to the Florideæ, the other denying the presence of sexual organs and tracing the development of the compound sporocarp through an asexual line of ancestry.

Throughout the Phycomycetes there remains little doubt of the existence of a distinct sexual process. Representative forms in the different families have been described by De Bary (1) and many others. The structure of the sporocarp, and origin and development of the spore in this group are comparatively simple processes, and have been more or less thoroughly demonstrated. Nuclear phenomena here have also received some attention. Among the later and more complete articles may be cited that of Istvánffy (2) treating of numerous different species.

The Mesomycetes (Brefeld) seem to lend themselves to either line of development. Those leading toward the Ascomycetes furnish some instances of undoubted cytoplasmic fusion at least, while in the Ustilagineæ and Uredineæ Dangeard (3) and

Sappin-Trouffy (4) describe certain nuclear fusions which they explain as sexual processes. No details of nuclear structure and division in these forms have as yet been described.

In the Basidiomycetes the only process analogous to conjugation is the fusion of nuclei in the basidium before the formation of spores. Nuclear studies in this group have been more numerous and complete than elsewhere among the fungi. De Bary (1) in three species has observed the presence of nuclei in the basidium. Rosenvinge (6) has demonstrated the same in thirty-five species. Strasburger (7) has observed the nuclei in Agaricus and found that they divide. Wager (8) in 1893 published the results of extensive nuclear studies upon Agaricus and Amanita. According to his statements, the nuclei of the basidia fuse in pairs before spore formation, and after this fusion successive bipartitions of this fused nucleus occur to furnish nuclei for each of the four sterigmata. During division the nuclear membrane is gradually dissolved and the nucleolus and chromatin masses left free in the cytoplasm. The latter have previously arranged themselves in an equatorial plate. A spindle is now formed, at the poles of which are dark rounded bodies, probably centrosomes, but he fails to find any radiating striæ. During the division the nucleolus disappears. After division of the chromosomes the spindle disappears and the daughter chromosomes at either pole fuse together and, he states, "apparently form the daughter nucleole." At the same time the linin network becomes more strongly differentiated, new membranes are formed, the daughter nuclei assume the size and appearance of the parent nucleus and are again ready to divide.

In the Ascomycetes minute study becomes much more difficult, the investigations are more meager and less reliable, and the conclusions are more at variance. Of the generalizations made by De Bary (1) and others it is only necessary to call special attention here to those on the sphæriaceous Pyrenomycetes. De Bary cites only Xylaria and Sordaria as having unmistakable sexual organs, and in these conjugation has not been observed. In Xylaria the archicarp, he says, seems to disappear before the

formation of the asci, and hence no relation can be traced between fertilization and the origin of the spore. In Sordaria also, according to Woronin (9), the origin of the ascus cannot be traced certainly to the archicarp. "In Claviceps, Epichloe, Pleospora, and perhaps also Nectria, no cooperation of the above named organs (archicarp and antheridium) has been observed, and no distinct ascogonium." . . . "The young perithecium, as at present known, is a body consisting of similar hyphæ or parenchymatous cells, and its elements are gradually fashioned and differentiated into the parts of the perithecium."

"Hartig's (10) conjecture with regard to Nectria, that special ascogenous initial organs are really present on the very young stoma, but up to the present time have been overlooked, may certainly hold good of Claviceps and Epichloe." (De Bary, *l. c.* 200.) Hartig (10) finds sexual organs in Rosellinia. Woronin (9) claims sexuality for Sphæria and Sordaria, and Harper (5), in a recent article on Sphærotheca, shows conjugation and traces the origin and development of the ascus from the fertilized archicarp.

Thus far we have shown strong support for the theory of the presence of sexuality in the Pyrenomycetes. But Brefeld (11), with a school of well known workers, opposes this view, tracing the development of the compound sporocarp through an asexual line and denying the significance which the De Bary school attach to certain characteristics of these plants.

Concerning the nuclei of the Ascomycetes we learn from De Bary that the young asci in both Discomycetes and Pyrenomycetes, so far as studied, contain each a primary nucleus, and later one smaller nucleus is present in each of the eight spores. Sadebeck (12), in 1883, published certain details of nuclear behavior in the Exoascaceæ. He indicates that karyokinetic divisions take place in the ascus, increasing the number of nuclei from one (the primary nucleus) to one for each of the spores. His karyokinetic structures are, however, very rudimentary, and are represented in his figure by two rounded granular bodies at the poles of a spindle consisting merely of three lines. No details of karyokinesis are given.

Fischer in 1885 goes somewhat more deeply into detail regarding the structure of the nucleus in Exoascus. According to his observations the nucleus is a round granular mass in which smaller and darker rounded bodies appear just before division. Next a spindle is formed, consisting of four threads converging slightly toward the poles. In the equatorial plane of the spindle four chromosomes now appear, which divide and pass to the poles of the spindle, where they form themselves into daughter nuclei. This description is of course crude, as were the methods of observation upon which it was based. Gjurasin (13) in 1893 published the first detailed account of karyokinesis in the Ascomycetes. It is based upon a study of *Peziza vesiculosa*. He describes the nucleus as consisting of a distinct round granular nucleolus surrounded by a layer of hyaloplasm in which an indistinct network is present. At the time of division the nucleus elongates, the nucleolus becomes eccentric, the chromatin aggregates into small masses, and two centrosomes with radiating striæ appear. Between these are drawn the nearly parallel threads of the spindle. The chromatin masses collect in the equatorial plane of the spindle and divide, and the daughter chromosomes very quickly seek the poles and soon are surrounded by new nuclear membranes, the membrane of the parent nucleus having meantime disappeared. In these daughter nuclei the nucleoli appear after the formation of the membrane, and with their appearance the mother nucleus, which up to this time has persisted lying free in the cytoplasm, disappears. Second and third divisions occur in like manner. Gjurasin states that he spent two years in the search for these karyokinetic structures, obtaining his final results by means of the Hermann and Flemming methods of fixing and staining.

Harper (5) in 1895, by methods similar to those adopted by Gjurasin, observed karyokinesis in *Peziza Stevensoniana* Ellis, and *Ascobolus furfuraceus* Pers. From studies of these and various allied Discomycetes he concludes that the members of this group are especially favorable for the study of nuclei in the ascus. The results of his work may be summarized as follows:

there exists in the young ascus four nuclei which fuse in pairs to form a single primary nucleus. This nucleus divides in a true karyokinetic manner to furnish a nucleus for each spore. In the process of division (1) the chromatin collects in masses on the network; (2) the nucleolus becomes eccentric; (3) centrosomes with radiating striæ appear; (4) the chromosomes arrange themselves in the equatorial plane, divide in halves and seek the poles; (5) the nuclear membrane is ruptured and the threads from pole to pole become very much elongated and parallel. The nucleolus meantime has gradually diminished and by the time the daughter nucleoli are formed it entirely disappears. The daughter nuclei attain normal size and structure and undergo two further successive divisions, providing one nucleus for each spore.

The foregoing summarizes briefly the work so far done along the lines of morphology and development in the higher fungi. As may be seen, the first two divisions of the subject (sexuality and structure of sporocarp) have received a comparatively large share of attention, but the conclusions even here are contradictory and unsatisfactory. Spore development and nuclear phenomena have been worked out to some extent in the Basidiomycetes and Discomycetes, Harper's work on one genus stands alone for the Perisporiaceæ, while nothing at all has yet appeared for the sphæriaceous Pyrenomycetes. Hence certain members of the last named group have been made the basis for the investigations of which the results are here presented.

METHODS.

Growing.—The material used for study was grown in artificial cultures and examined either in sections or by growing it upon glass slips immersed in nutrient media and transferring these slips directly to the stage of the microscope. The latter method served as a check on the former, since in sections the numerous cut ends of the mycelium are likely to lead to erroneous conclusions. As soon as pure cultures of the desired species were obtained by ordinary dilution methods, the spores were sown in

infusions of bean stems or mushrooms in Petri dishes. In these dishes had been placed carefully sterilized glass slips and cover-slips. The spores germinated sometimes upon the slips, sometimes floating free in the liquid, in which case the colonies rested upon the surface. Where they germinated on the slips, the mycelium adhered closely to the glass and made the process of fixing and staining much less difficult. On the other hand, the colonies which floated on the surface of the liquid seemed to take on more natural characteristics and they could be lifted out on a slide and prepared for the microscope. Many of the colonies thus grown developed mycelium in such abundance as to make observation very difficult. This was especially true of colonies grown in mushroom infusion, which seemed a most favorable medium, but it was also true that in these dense colonies the fruit was most abundant. The material for sectioning was grown in potato agar slightly acidulated to suppress bacteria.

Fixing and staining.—Slides bearing colonies were lifted carefully from the Petri dishes at different stages in their development and washed in water. Various methods of fixing and staining were tried. The most satisfactory was to fix with a cold 1 per cent. acetic saturate solution of mercuric chloride, stain with alum-eosin and mount in glycerine. The mercuric chloride fixes this material almost instantly and when washed off well with water leaves it in condition to take a clear decisive stain in the alum-eosin. This stain does not bring out nuclear structures with great distinctness, but cell walls come out sharp and clear. It stains the young mycelium and perithecia almost instantly, the latter taking a slightly deeper stain than the former. Good results were also obtained by staining with carmalum the material fixed in this way. Ehrlich-Biondi, hæmatoxylin and fuchsin were also found fairly good. The efficiency of any stain was found to depend largely upon the age of the fungus. Young mycelial threads stain very readily, while the very mature ones refused the stain altogether.

Sectioning.—Small blocks of potato agar containing colo-

nies were cut out from the Petri dishes, dropped for a few minutes into the mercuric chloride and then allowed to wash in running water over night or at least for six to eight hours. In this process much of the agar was washed away, but the colonies were sufficiently compact to hold themselves together. They were then passed through successive alcohols, infiltrated and embedded in collodion, and cut with a microtome in sections from 6μ to 12μ thick. No other fixative tried seemed to leave the material so susceptible to stains as the mercuric chloride. Among others used may be mentioned chromic acid, Flemming's stronger solution, Hermann's solution, and Fish's picro-aceto-sublimate. The solutions of Flemming and Hermann were perhaps equally good when cleared with hydrogen peroxide, but without this additional trouble the sections so fixed were useless because of the discoloration of the protoplasm. With Fish's mixture some very good sections were prepared, but they were in no way superior to those fixed in the more simple corrosive sublimate.

In all the earlier part of the work the sections were stained with Mayer's carmalum. This produced no differentiation of nuclear structures, and much time was spent in experimenting with various stains and combinations of stains in the hope of getting a color differentiation. This was not accomplished, but in the later work upon nuclei Hermann's safranin-gentian-violet method as given by Zimmerman (17) was employed with very satisfactory results. Gjuarsin's methods as given in his article on Discomycetes were also found valuable.

The greater part of the observations were made with a Bausch and Lomb microscope, one-twelfth inch objective and one inch ocular, but for nuclear study this was found insufficient, and a Zeiss instrument with a one-twelfth inch objective and no. 8 ocular was employed.

TEICHOSPORA.

This fungus was found growing upon dead branches of oak in the vicinity of Ithaca, N. Y., late in November 1895. The

perithecia were then past maturity and many had discharged their spores. Closely associated with the perithecia were numerous pycnidia, appearing as much smaller rounded black bodies. A dilution culture was made in potato agar from the ascospores which were supposed to be free from the pycnospores. The ascospores soon germinated and camera lucida drawings of the germinating spores were made (*fig. 1*). Numerous colonies now appeared in the plates. They were circular, showing radiations from the center, and in concentric rings, a little later, the beginnings of perithecium like bodies appeared. These colonies were then transferred to infusions of bean stems in tubes where they continued to develop. Contrary to expectation, however, when the round black perithecia like bodies matured they contained not asci, but pycnospores. The pycnidia were rounded or oval in form, opening usually by two apertures (sometimes by more, sometimes by only one), and extruding their spores in the typical worm like manner. The spores are fusoid-elliptical, hyaline, continuous, about half the diameter of the mycelium. In the colonies the pycnidia appear first near the center and may be found in all stages of development along a radius of the colony, the youngest stages nearest the circumference. The beginnings of these structures are shown in *figs. 4* and *5*. No pycnospores had been observed in the agar plates in which the sowing was made, and since the ascospores were known to have germinated, the natural conclusion was that ascospores had produced pycnidia, but this conclusion was found to be doubtworthy later. Meantime, there had appeared in the bean tubes, associated with these pycnidia, a grayish white mycelium with erect branching hyphæ upon which were borne multiseptate conidia (*fig. 6*). The hyphæ were closely septate and the spores varied from the diameter of the hyphæ, when they were hyaline or yellowish in color, to twice that diameter as they became dark brown. Although these were normally borne on the tips of hyphæ, as true conidia, they sometimes appeared as intercalary growths in the mycelium. When these conidia were quite mature a sowing of them was made in potato agar. Colonies

soon began to form around the spores, but whether the growth began from the germination of the spores or from attached fragments of mycelium was not determined. As only pycnidia and conidia were produced from these sowings, the cultures were abandoned. Sowings of pycnospores were next tried, but in several successive generations only pycnidia and gonidia appeared and there seemed no hope of reproducing the ascigerous fruit. This failure of the ascospores to reproduce themselves even in alternate generations seemed unnatural and another culture was attempted from the original material. This had meantime been kept in the laboratory and had become old and dry and resisted all efforts to make it germinate. On January 25, 1896, some fresh material was collected by Professor Atkinson and from this ascospores were obtained which germinated readily in potato agar. Certain spores in the plates were marked and watched carefully until February 1, when they had attained distinctive characters and the numerous other colonies in the plates could be safely and certainly identified as the same. They appeared to the naked eye as dark spots with a light radiating fringe. With a carefully sterilized scalpel transfers of these colonies were made to bean stems in tubes. Ten such tubes were prepared. The transfers were made in a close culture-room and with the utmost precaution to avoid contamination. There was no doubt that these colonies grew from the ascospores, but probably none of these colonies developed. For a long time no growth appeared. Finally, in two tubes, a few pycnidia were found, and in one or two other tubes non-related forms appeared. In the plates from which the ten transfers had been made there were found (Feb. 3) a few colonies much in advance of those which had been traced from the ascospores, but very strongly resembling the pycnidia bearing colonies of previous cultures. These, however, contained the beginnings of perithecia in some of which asci had already developed. These colonies had apparently grown from fragments of mycelium or of the perithecia. They were removed piecemeal at short intervals of time (about three hours) and fixed and embedded for sectioning. It

was hoped by this means to get all the successive stages of development (see account of sections below). Transfers of these larger colonies were also made to the bean stems in tubes, where they continued to develop perithecia and March 5 a culture was made from the ascospores contained in these perithecia. These spores, although full grown, were still hyaline in color, but they possessed unusual vigor. The colonies grew very rapidly and developed to maturity. Perithecia only were produced and the same was true in a third generation of this series. Another culture was now made from the pycnidia and conidia but again they produced only their own kind. This failure to connect the ascospores with the other forms led to the conclusion that they are probably distinct forms. The ascospores obtained from the oak in the fall germinated but failed to produce thrifty colonies, probably because they had lost vitality or because they were not readily adaptable to artificial conditions. Those sown in the first cultures very probably germinated and then died, as did those transferred to the ten tubes. The pycnidia found in these first cultures no doubt originated from pycnospores which were associated with the asci but overlooked in making the culture. This might easily have been the case, since the pycnospores are very small and scarcely distinguishable in potato agar. The study of the pycnidia and conidia has been included here because of interesting points of resemblance in the development of the pycnidium and the perithecium, and also because in nature the two forms are found so intimately associated. Indeed their connection is not disproved, although it has been impossible to establish artificially a life cycle including the three forms.

The ascigerous colonies which were finally obtained started, as has been said, from fragments of the mycelium or perithecia, and the ascospores thus obtained, being fresh and vigorous and perhaps also somewhat inured to conditions, germinated readily and reproduced themselves. This is further evidenced by the fact that during the winter two or three attempts were made to get cultures of the following species from herbarium specimens: *T. trimorpha*, *T. fusispora*, *T. aspera*, and *T. nitida*. The first named

species had been in the laboratory but a few months and its spores germinated but developed no further. The others were older and refused even to germinate.

Attention was now confined to the ascigerous colonies of the species in hand, and with methods previously described the following observations were made. The mycelium is composed of septate threads, each cell of which contains several nuclei. With alum-eosin and carmalum stains, the nuclei are distinguishable merely as round points stained more deeply than the cytoplasm and lying at or near the center of a circular clear space. In the mycelium nothing more minute than this could be determined in regard to the structure of the nucleus. None of various stains tried succeeded in differentiating any elements. Any stain which affected the nucleus also attacked the protoplasm of the cell. The nuclear membrane was usually sharply defined as a dividing wall between the clear circle and surrounding cytoplasm.

At various intervals in the mycelium certain cells were found which were more or less swollen (*fig. 7*). The protoplasm in these was more dense than in the adjoining cells as shown by the deeper staining. This over-staining made observations upon nuclei in these cells very uncertain, but the cells proved to be the beginnings of perithecia. The first dividing wall is thrown across parallel to the septa which delimit the original cell (*fig. 8*). Each of the two daughter cells then divides by a wall perpendicular to this, forming a four-celled spherical body. As this grows, further divisions occur somewhat irregularly (*figs. 9* to *11*) until we have a body consisting of a solid mass of irregular cells as yet without any differentiation. Sometimes at this stage the young perithecium is surrounded by a single layer of mycelial threads which have arisen in the neighborhood of the original cell and interwoven themselves, forming a sort of wall for the perithecium. The wall proper in this species is not more than a single layer of cells thick and in many cases it seems to be formed by the thickening of the cell walls of the outer layer of the spherical mass, without the assistance of any surrounding filaments. It does not become dark and hard until the perithe-

cium is nearly or quite full grown. When the growing perithecium has attained a little more than half the normal diameter of the mature fruit there may be seen at its center an oval sac containing a single nucleus and filled with densely granular protoplasm which stains very deeply. This sac is simply a swollen cell of the interior of the sphere. Simultaneously with its appearance a part of the loose parenchymatous tissue surrounding it breaks down so that the young ascus lies in a more or less disorganized, gelatinous mass. Very soon the other asci appear one by one. In many cases the sections showed two large ovoid sacs lying side by side and almost filling the interior of the perithecium. The later asci developed as the growth of the sphere made room for them.

Earlier investigators have attempted to find the connection between the origin of the asci and a supposed fertilized archicarp (see Woronin, De Bary, and Hartig, *l. c.*). In this case there exists no probability of a process of fertilization. In many cases the entire sporocarp may be traced from a single cell around which no other filaments are present even to take part in the construction of the wall, and in cases where extraneous filaments are applied to the archicarp it happens after several divisions of the original cell have taken place. If we consider the single swollen cell in the mycelium as the mother cell of the entire perithecium it is a question of some interest what determines which of the daughter cells become asci and which are disorganized. Harper (5) finds in Sphærotheca a structure consisting of from five to seven cells, arising directly from the fertilized archicarp, and a certain one of these containing two nuclei and giving origin to the ascus. We might consider the entire cellular structure of the fruit of Teichospora homologous with this five to seven-celled growth in Sphærotheca, in which case we should expect to find in the former certain binucleated cells functioning as mother cells of the asci. This may indeed be true but the observations so far made do not warrant such a statement. It is only in exceptional cases that the nuclei in these cells can be distinguished at all, on account of the density of the proto-

plasm and consequent deep staining. In the earlier stages, when the structure consists of two to four cells, faint outlines of nuclei can sometimes be seen, and in one or two cases division was suspected, but no positive statements are warranted regarding the nuclei in the perithecium prior to the formation of the ascus. As soon as the ascus is differentiated, however, the nucleus becomes very distinct. No fusion has been observed, and in the youngest stages each ascus contained one large clearly defined nucleus. This nucleus is much larger than those found in the mycelium, but is otherwise similar.

That some process of nuclear division takes place is apparent. As before stated, the young ascus contains a single nucleus. This has been called by De Bary the "primary nucleus." It does not, however, as he says, lie always in the end of the ascus. Its position is variable, but it is found oftenest near the center of the ascus and in a bed of very dense protoplasm. Its diameter is often equal to half that of the ascus. Neither does this primary nucleus disappear and eight daughter nuclei appear simultaneously, as has been stated. All the successive stages of division have not been observed, but an ascus containing two nuclei was found, and another with eight. In the latter case they were arranged in pairs, the two largest having moved a greater distance apart than any other pair (*fig. 19*). This, with the further evidence about to be given, was considered sufficient proof that the eight nuclei are provided by successive bipartitions of the primary nucleus. On this point the ascus next to be described was indirectly very instructive (*fig. 20*). In its protoplasmic content could be discovered, by careful focusing, the outlines of eight spores. The protoplasm in the spores was more dense than that surrounding them, and hence they were stained more deeply. The two spores nearest the base of the ascus contained one nucleus each and were not divided. A third spore contained two nuclei, lying near the ends of the spore, but no dividing wall had yet appeared. Four other spores contained two nuclei each, situated at various distances from a centra septum. This septum was unstained, appearing like an open

space in the protoplasm, but was more distinct than the outlines of the spores. The eighth spore of this ascus was in such a position that only part of its outline could be seen. In another ascus (*fig. 21*) there were found eight well defined spores, the walls being now distinct. Seven of these were divided by single central transverse walls into two cells each. Each cell contained a single nucleus. The eighth spore was bent upon itself in such a way as to conceal one end and only one nucleus was visible in it. There can be little doubt, however, that another was present in the concealed end, and it may be asserted safely that at this stage the ascus contains uniseptate spores having a nucleus in each compartment. The septa in this case had been formed soon after the first nuclear division in the spore. In preparations from material slightly more mature, spores were found in which the number of nuclei varied from two to ten, corresponding in number usually to the number of cells in the spore. An exception to this occurred in the case of one entire perithecium (*fig. 16*). Here each spore contained from four to ten nuclei but no dividing walls could be seen. Either the staining was at fault, which seems improbable, or the formation of the wall does not always follow immediately upon nuclear division, but takes place instead after all the successive bipartitions of the nucleus are complete.

The foregoing evidence, together with the fact that the nuclei occur constantly in pairs throughout the mycelium as well as the fruit, seemed conclusive proof that nuclear division does take place, but for a long time no details of the process of division could be discovered. Later, cases were found in which the hyaline court had become elliptical in outline and contained sometimes one eccentric dark spot, and sometimes one at each end of the ellipse. These at first seemed very similar, and the nucleus in this condition corresponds closely with Trow's (14) figures of what he calls "fragmentation" in the nucleus of Saprolegnia. If his theory be correct then the central stained spot is probably a chromatin plate, and by simple fragmentation furnishes a half plate for each daughter nucleus, and this grows quickly to full size. This must be based upon the assumption that the two

stained bodies at the foci of the ellipse are similar in structure. This indeed seemed at first to be true, since no differentiation resulted from the use of the ordinary differential stains. On the other hand, it seemed quite as possible and more probable that the original dark spot of the nucleus is the nucleolus. In a resting condition this alone is visible, but preparatory to division the nucleolus moves off to an eccentric position, the hyaline court elongates, and the chromosomes accumulate to form the second chromatin mass in the opposite end of the ellipse. With more exact staining methods and greater magnification this possibility proved to be the fact. The resting nucleus contains a large nucleolus in the center of the cell sap. In this sap in some cases can be seen an indistinct linin network of very fine threads (*fig. 27*). Preparatory to division the nucleolus becomes eccentric and the circular space elongates. The linin now becomes more distinct, the threads having grown thicker at certain points (*fig. 28*). In the next stage small deeply stained chromatin bodies (not more than four have been counted) appear at the end of the ellipse opposite the nucleolus, arranged in what might be the equatorial plane of a spindle, but no spindle threads have been distinguishable in any case. At the poles of this plane were two small rounded bodies which were probably centrospheres, although no radiating striæ could be seen (*fig. 29*). Again, two groups of chromosomes, of four each, were found near the positions previously occupied by the centrospheres (*fig. 30*). By this time the nuclear membrane had become partially dissolved. The nucleolus was now vacuolated and less deeply stained and lay partially out in the surrounding cytoplasm. The cell sap was still present but constituted a somewhat distorted mass, and in it were the outlines of two new hyaloplasmic circles each surrounding a group of daughter chromosomes. In the next stage the ascus contained two new nuclei, somewhat smaller than the original but having the same structure (*fig. 31*). No secondary division was observed. Upon these cases, which have been illustrated from camera lucida drawings, may be based the conclusion that the nuclei in these asci divide karyo-

kinetically. The failure to discover the spindle may have been due to inadequate staining or insufficient magnification. In other essential details the process agrees with Harper's description of karyokinesis in the Discomycetes. If the details seem incomplete it must be remembered that the largest nuclei measured only five or six micromillimeters in diameter, and minute structure was extremely difficult to determine with the instruments available.

The general sequence in the process of the development of the ascigerous fruit of Teichospora may now be summarized as follows:

1. A single cell of the mycelium by successive divisions and growth forms a solid sphere of parenchymatous tissue.

2. Certain of the interior cells of this tissue become enlarged and differentiated into asci.

3. Each ascus contains at first a single large nucleus, which by successive karyokinetic divisions furnishes a single smaller nucleus for each compartment of the multiseptate spores.

TEICHOSPORELLA.

An undescribed species of Teichosporella, found by Miss Stoneman on oak and studied in a similar way, was found to develop in almost exactly the same manner as the Teichospora. So similar are the steps that it seems unnecessary to repeat the description in detail. A few figures are given showing the early stages of the perithecia (*figs. 7a, 10a, 11a*) as confirmatory evidence of the rather unusual way in which the sporocarps in this group arise, and also to call attention to a slight peculiarity which may have some significance in the question of phylogenetic relationships. This peculiarity is shown at *x* in *figs. 10a* and *11a*. It consists of a protuberance in the region of the archicarp, which by its shape and position suggests the possibility that it may be a degenerate rudiment of an antheridium. This feature is quite constant, and in general appearance not unlike the antheridia found later in Ceratostoma. If this explanation of its presence be accepted, then this genus furnishes a connecting link between

forms having more or less complete sexuality and the Teichospora where all trace of an antheridium has disappeared.

Teichosporella exhibits another peculiarity in the development of the ascus. Instead of an evenly granular protoplasmic content filling the ascus from the time of its origin, there exists here, at first, an apparently empty sac formed, not by the swelling of a cell as in Teichospora, but as a papillate and then somewhat inflated outgrowth from a cell. Into this sac the protoplasm seems to push its way through a comparatively narrow opening at the base. The process may be understood best by reference to *figs. 22* to *26*, in which successive stages of the process are shown. The same conditions were found in the living material, hence it could not have been due to the action of reagents. The appearance may be due to an abnormal swelling of the ascus wall which recedes with its growth, or to the presence in the young ascus of a non-chromatic plasma or cell sap. At certain stages a zone of this same colorless sap may be seen enveloping the spores (*figs. 25* and *26*) after the ascus itself has become filled with the normal protoplasm.

The nuclear processes in this species agree, so far as studied, with those in Teichospora, but the work on this form was not repeated with the better stains and higher magnification used in the later work on Teichospora.

The development of the sporocarp as above given for these two genera is indeed widely different from any process heretofore described for the perithecia of any of the Pyrenomycetes. It is interesting to note, however, that it corresponds very closely with what has frequently been described as the normal course of development of pycnidia. This and the apparent loss of sexuality here suggests that these may be more degenerate forms than some others, and further that extreme degeneracy leads to the production of pycnidia only, these last named fruit forms being merely reduced perithecia.

CERATOSTOMA BREVIROSTRE.

This fungus was found growing upon decayed mushrooms in a garden near the university. In artificial cultures it produced

fruit so rapidly and in such abundance that it seemed a specially favorable subject for developmental study. The large dark brown spores in germinating extruded an endospore through a tiny pore at the more pointed end of the spore. The endospore contained a large vacuole and many nuclei, and from it arose one or more germ tubes (*fig. 32*). These germinating spores have been drawn large to show the nuclei, which are very distinct, occurring usually in pairs, but so small as to require great magnification in order to be seen at all. Spores sown in potato agar or upon bean stems produced colonies of whitish mycelium, sending out from the center strong primary radiating filaments which become plumose at their tips.

The colonies spread flat upon the substratum, and when grown in tubes the radiating threads pushed far out upon the glass, bearing fruit at some distance from the medium upon which the spore had germinated. This fact proved of great advantage in the later microscopic study, since the thin weft of mycelium grown on slides in Petri dishes could be easily transferred to the stage of the microscope and afforded excellent opportunity for study of the material in the best possible condition. The mycelium was comparatively scanty and no conidia or pycnidia appeared at any time during the many successive cultures that were made. The necks of the perithecia furnished a noticeable example of heliotropism. Those grown in tubes standing in boxes, and hence lighted only from above, turned strongly upward. A tube was then wrapped in dark paper, leaving only about an inch at the bottom exposed to the light, and suspended by a string. In this the necks turned sharply downward. Those grown in plates inclined always towards the window.

The first microscopic study was directed specially toward determining, if possible, whether there exists here any sexual organs such as have been described by Woronin (9) for the closely related genus Sordaria. For this purpose the fungus was grown upon bean stems and prepared for sectioning in collodion. Various methods of fixing and staining were tried, but nothing con-

cerning the earliest stages of the perithecia could be discovered. Colonies grown in agar were next tried. These were fixed, hardened, and embedded in much the same manner as those grown on stems. This also proved unsatisfactory. The cut ends of the mycelial threads were deceptive, and although some very suggestive features appeared no positive conclusions could be drawn. An attempt was next made to study the colonies in cell-cultures and in the agar plates in which they grew. The latter proved more nearly successful than any of the former methods, but still much was concealed by the cloudiness of the agar and the fact that few colonies grew close enough to the surface to be focused upon with the higher power objectives. Finally the plan of growing on glass slips was adopted and gave the desired results. Slides bearing colonies were removed from the dishes at successive short intervals and treated as before described. The greatest caution was necessary to keep the colonies *in situ*. Unless they had grown very close to the slide they were speedily tumbled into a tangled, shapeless mass by the action of the fluids. The colonies which grew floating in the liquids required endless patience in preparation, but in this delicate material, when it was successfully prepared and mounted in glycerine, the long sought beginnings of perithecia were finally discovered. They occurred in such abundance as to leave no doubt of their function. They were noticeable first as deeply stained spots scattered thickly through the mycelium and varying in size from a little more than the diameter of the mycelium to one-fourth that of the diameter of a normally mature perithecium. This amount of variation in size could be seen usually in a single preparation, and so slight and uniform were the gradations from the larger to the smaller that there remained no doubt of the two being identical structures. The smallest of these bodies consisted of a short swollen branch arising from a primary branch of the mycelium and immediately becoming curved. From this short slightly curved branch (*fig. 33*) to the several coiled type (*fig. 34*) all intermediate stages were found. The size and shape varied greatly, and from the various assortment only the more typical forms

were chosen for illustration. In most cases, an antheridial branch in contact with this curved structure (which was evidently the archicarp) could be plainly seen. This was a slender branch arising usually, though not always, from another filament and applying itself to the archicarp. In some instances the antheridia traveled comparatively long distances to reach the archicarps, and some were found coiled once or twice upon themselves in their courses. In most cases the two gametes were so interwoven that they could not be exactly traced, but in two instances unmistakable fusion of antheridium with archicarp was discovered (*figs. 35* and *36*). In both cases the antheridial branch is applied just at the tip of the female organ and the walls of both at the point of contact are dissolved. Many nuclei are usually present in both gametes, but they have not been seen fusing. The antheridium does not lose its protoplasmic content. It is usually less deeply stained than the archicarp, but this is also true before the two organs meet, and means probably only that the latter is richer in protoplasm than the former. The antheridium, moreover, is not always present. Numerous instances occurred, as shown in *fig. 33*, where the archicarp seemed to be developing without fertilization. There is, of course, no positive proof that these non-fertilized cells would ever produce asci, but the evidence given by the older stages of these points very conclusively toward the existence of parthenogenesis. Other peculiarities such as those shown in *figs. 37* and *38* were very interesting. In *fig. 38* the antheridium, while certainly present, has turned entirely away from the female branch, while the latter has continued to coil very much beyond the limit at which fusion usually takes place. *Fig. 33* also has become septate in accordance with the normal plan of development after fertilization. *Fig. 37* shows a tendency in another direction. Here the archicarp, having failed of fertilization, has been produced into a vegetative filament in a manner suggestive of what the writer (19) finds occurs at times in Vaucheria. These vegetative outgrowths were comparatively rare, but still were of sufficiently frequent occurrence to demand attention. They might indeed

be caused quite as easily by insufficient nutrition as by lack of fertilization.

About the time that fusion of the two gametes occurs, in normal cases numerous branches arise in the neighborhood of the young fruit and become intimately interwoven around the sexual organs to form the wall. For a time the swollen archicarp can be seen in the center of this knotted mass of sterile filaments, but as the wall thickens and the threads of which it is composed become more closely septate this interesting structure is lost from sight. For further steps in the process it is therefore necessary to refer to sections. To get very early stages it was necessary to fix the growing material before any sign of color appeared on the outside of the tiny rounded bodies which were just becoming visible in the mycelium. The rate of growth varied so much that no definite age could be established as the proper one for sectioning, and repeated trials were made before sections containing the desired information were secured. By means of a long series of observations it was at last determined that in the stage just preceding the origin of the asci, the perithecium consists of a spheroidal mass of cells of three kinds. First is an outer layer two or three cells thick of thick-walled, nearly isodiametric cells, made up of the sterile hyphæ which envelop the young sexual organs. Inside this is a layer, two to three cells in thickness, of tabular cells which appear to have been laterally compressed by growth from within. These two have evidently been formed from the enveloping hyphæ. The center of the sphere is entirely filled with loose, spongy tissue composed of parenchymatous cells, well filled with protoplasm. These cells show no differentiation of form, and nothing exists to indicate where the asci will originate save that in certain sections a small group of these cells, lying about midway between the center and circumference of the spongy tissue on one side, takes a slightly deeper strain. Even in the very young stages no sign of the sexual organs imbedded in the spongy tissue could be found. It is at this point that De Bary and others have been obliged to leave gaps in their records of perithecial devel-

opment. De Bary (1) says of Xylaria that the hyphal coil, or "Woronin's hypha" as it has been termed, seems to disappear before the formation of the asci begins. Hartig (10), in his study of Rosellinia, states that the asci seem to spring from a certain gelatinized zone, but whether they originate in the sexual apparatus or in the *Wandparenchyma* he is unable to determine. Woronin (9), too, fails to trace the exact origin of the ascus in Sordaria or to find the connection between it and the "hyphal coil." That a structure so prominent as this sexual apparatus, if it persists throughout the later development, should elude the notice of so many observers is quite incredible. It seems quite as improbable that this fertilized archicarp should fail to play any part in the production of the ascospores Harper (5) finds in Sphærotheca that the oosphere grows out into a branch of five to seven cells, from a certain one of which the ascus arises. The existence of the sterile cells of this branch suggests a solution of the problem in the true Pyrenomycetes, which has already been partially verified in the case of Teichospora, and is further confirmed by what is found here. Before the enveloping weft of threads, which are to constitute the wall, becomes too dense it may be observed that the coiled archicarp is becoming septate. Even in very young stages this septation has gone so far that in section the interior of the perithecium appears as a homogeneous mass of cells in which the outline of the coil cannot be distinguished. It now seems logical to conclude that this entire mass of parenchyma which forms the interior of the immature perithecium is formed by successive cell divisions in the archicarp. Instead of the five to seven-celled branch, giving rise to one ascus in Sphærotheca, we have here an indefinite number of cells giving rise to a variable number of asci. This is strictly analogous with the process in Teichospora, which was more easily traced because of the absence there of enveloping threads. It is in no way contradictory to the observations of Woronin, De Bary, and Hartig, though it fills in the gap which they have left open. It is true that only a comparatively very small number of these cells give birth to asci and the question as to what determines the fertile cells is still

open. Here, as in Teichospora, no binucleated cells were discovered, but it was also true, as in the former case, that either because of the density of the protoplasm in these cells, or because of some fault in manipulation, no nuclei at all were discovered in any of these cells. As has been said, the only differentiation is a slightly deeper stain in a certain group of cells, the position of which corresponds with that from which the asci arise. It may be argued that only this group of deeply staining cells are formed by division of the archicarp, but if this were true we should certainly expect in some cases to be able to trace the outlines of the fertile hypha. Moreover this latter theory would fail to account for the existence of the sterile cells. The marked difference in structure between these cells and those of the wall make it highly improbable that both arise from the enveloping hyphæ. Then, too, when the sections become mutilated by the rolling of the collodion or by rough handling it is a noticeable fact that this central tissue breaks out intact, leaving the inner surface of the wall quite smooth. This theory of development tends also to harmonize what first appeared to be a very peculiar condition in Teichospora. The archicarp in Teichospora is not fertilized and is not specialized in form, but the further processes of development are essentially homologous. There remains only to prove that the mother cells of the asci contain two nuclei which fuse before entering the ascus in order to establish a line of development analogous not only to that described for Sphærotheca but also to what is found in many cases in the Florideæ. This intervention of vegetative tissue between fertilization and the production of spores, provides a sporophyte phase in the life cycle of these fungi which has not hitherto been known.

From the deeply stained group of cells in the central parenchyma the asci arise as papillate outgrowths, densely filled with protoplasm and staining much more deeply than the surrounding tissue. Their bases lie close together and the asci, which are long narrow-cylindrical and very numerous curve upward toward the ostiolum. Their protoplasm is densely gran-

ular and usually somewhat vacuolated. As they grow they become slightly constricted at the base. Certain ones advance more rapidly than others, so that in a single perithecium all stages of development may frequently be seen. The "bouquet" of variously sized asci lies embedded in the loose surrounding parenchyma, a part of which persists until the asci are quite mature. A distinct layer of this tissue is present between the bases of the asci and the basal part of the wall. In the center of the perithecium, between the asci, these vegetative cells become disorganized and probably furnish nourishment for the growing asci.

Meantime the wall has also undergone some changes. With the growth of the asci the entire sporocarp enlarges, the cells of the outer wall become thicker walled and darker, while those of the inner wall undergo still more lateral compression. At a point opposite the base the ostiolum begins to appear. Its beginning is marked by a slight protuberance, beneath which first the cells of the inner then those of the outer wall begin to separate schizogenetically. The ends of these elongated inner wall cells after separation round off and look like filaments converging toward the canal of the ostiolum. As the neck increases in length these filaments also lengthen and extend up into the neck canal parallel with its sides. The growth of the ostiolum continues after the development within is quite complete. Indeed the asci often are broken down, leaving the spores free within the cavity before the neck reaches its normal length.

The nuclei of the mycelium have already been noticed. They seem essentially the same in structure as those found in Teichospora, consisting, so far as could be seen, of a nucleolus in a clear circular court. The fact that no linin has been seen in the nuclei of the mycelium is probably due to the small size of these nuclei and the fact that they are seen through the wall of the mycelium. In the ascus the nuclei of Ceratostoma are smaller than in Teichospora and fewer details were made out. The drawings were reproduced free-hand, as those made from the camera lucida were too small for satisfactory illustration. In

these nuclei there may be seen a linin network of very fine threads within the hyaline court (*figs. 40* and *41*). The first step toward division is the elongation of the court. The nucleus then becomes eccentric and the chromatin collects in masses on the threads. Then the network disappears. In the next stage that could be found the chromosomes had evidently divided and were arranged in two groups at some distance apart (*figs. 40* to *42*.) No centrosomes or spindle figure could be certainly distinguished, though in one or two cases something very like a spindle figure was faintly visible. Many of the intermediate stages are wanting. What becomes of the nucleolus cannot be certainly stated, but enough of the steps have been observed to warrant the conclusion that there is here a karyokinetic division, the more minute details of which might be detected by higher magnification. The stages found are illustrated in *fig. 40*, nos. 1–4.

HYPOCOPRA.

As Teichosporella served to confirm the observations made on Teichospora, so Hypocopra served as a check upon Ceratostoma. The species studied was obtained from dung and separated by transferring with a sterilized needle first to dung infusions and then to bean stems, where it grew and fruited abundantly. From the bean it adapted itself readily to different media. The germination of the spores (*fig. 42*) was studied by transferring small quantities of the agar in which they grew to slides, where they were stained with Mayer's carmalum and mounted in glycerine. They did not, as in Ceratostoma, extrude the endospore. The protoplasm exudes through a tiny pore at the end of the spore and pushes out into a germ tube. The division and growth of the nuclei takes place with startling rapidity. Before the germ tube attains half the length of the spore, from four to eight nuclei have appeared. The colonies of mycelium grown in agar are characterized by the appearance of the first perithecia in a definite ring at a short radius from the center of the colony. The sexual organs are essentially similar to those of Ceratostoma. Some of the more typical forms are

shown in *figs.* *43* and *44*. The hyphal coil may be traced for a longer time here than in the other genus. In *figs.* *45* and *47* it is shown somewhat straightened out and several times septate. For want of time the study of this form was not carried through to the end, but enough observations were made to furnish convincing proof that the course of development is essentially the same as in Ceratostoma. The process is somewhat more complicated than in Teichospora, and may be summarized as follows:

1. The spores upon germination send out polynucleated mycelial threads which become septate, branch, and form circular colonies.

2. Upon the mycelium are borne short thick branches which become curved, or sometimes several times coiled, and function as archicarps.

3. Near these archicarps are usually found long slender branches, the antheridia.

4. The antheridia intertwine with the archicarp, their tips meet and fuse.

5. The archicarps in some cases appear to develop without fertilization.

6. The archicarp by growth and division furnishes the cells which make up the interior of the perithecium.

7. From certain of these cells of the interior the asci arise.

8. In each young ascus there is a single primary nucleus.

9. The primary nucleus divides karyokinetically and the daughter nuclei in the same manner, to furnish a nucleus for each spore.

10. Nuclear division probably continues within the spore after the formation of the spore wall.

11. The wall of the perithecium is formed from surrounding filaments.

The evidence furnished by the foregoing investigations tends o corroborate the theory of De Bary that marked analogies exist between the higher fungi and the Florideæ. In the Pyrenomy-

cetes we may expect to find sexual organs and sexual processes in different stages of degeneracy. In Ceratostoma we have distinct sexual organs, but the first sometimes develops without the fertilization of the archicarp. In Teichosporella there remains only a possible rudiment of an antheridium, while in Teichospora this organ has entirely disappeared.

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EXPLANATION OF PLATES XIV–XVI.

Figs. 1, 2, 3, germinating spores of Teichospora.

Figs. 4 and 5, young stages of pycnidia.

Fig. 6, conidia.

Figs. 7 to 12, successive stages in early development of perithecium of Teichospora; *7a*, *10a*, *11a*, early stages of perithecia in Teichosporella.

Fig. 13, later stage of perithecium of Teichospora in which certain cells have begun to differentiate into asci and contain primary nuclei.

Fig. 14, perithecium showing young asci.

Fig. 15, part of a perithecium with several asci each containing primary nucleus.

Fig. 16, fragment of a perithecium in which all the nuclear divisions in the spores seem to have occurred without the formation of dividing walls.

Figs. 17 to 21, successive stages in development of the ascus in Teichospora, showing nuclear phenomena.

Figs. 22 to 26, stages in development of ascus in Teichosporella. Magnification not sufficient to show nuclei.

Figs. 27 to 31, successive stages in nuclear division in Teichospora.

Fig. 32, germinating ascospores of Ceratostoma brevirostre.

Figs. 33 and 34, archicarps or beginnings of perithecia without attendant antheridia.

Figs. 35 and 36, archicarps with antheridia attached, showing fusion.

Fig. 37, unfertilized archicarp produced into vegetative filament.

Fig. 38, archicarp and functionless antheridium.

Fig. 39, young perithecium in which the archicarp is still visible in optical section.

Fig. 40, fragment from the base of perithecium in which asci have begun to appear; *w. p.*, wall-parenchyma; *t*, tabular layer or inner wall. Asci are numbered to indicate successive stages in nuclear division.

Fig. 41, further development of asci.

Fig. 42, germinating spores of Hypocopra.

Figs. 43 and 44, archicarps of Hypocopra with antheridia present.

Figs. 45 to 47, young perithecia in optical section showing enclosed archicarp.